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(54) Title: USE OF A CARBOHYDRATE BINDING DOMAIN IN BAKING

(57) Abstract

A bread improving composition comprising an effective amount of a cellulose binding domain in combination with an anti–staling enzyme, optionally in admixture with other enzymes, as well as the use of the composition in the preparation of dough and baked products.

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USE OF A CARBOHYDRATE BINDING DOMAIN IN BAKING

FIELD OF THE INVENTION

The present invention relates to a bread-improving composition comprising a carbohydrate binding domain in combination with an anti-staling enzyme as well as to a method for retarding the staling of bread and similar baked products by use of the composition.

BACKGROUND OF THE INVENTION

It is generally recognised that there is commercial value in extending the shelf life of baked products by retarding the staling of such products. Consequently, much attention has focused on developing methods for preparing baked products, principally bread and bread-like products, which demonstrate an increased resistance to staling.

The mechanisms underlying the biochemical interactions between the components of bread which result in staling are yet to be clearly established. However, one factor believed to be of significance is the process of retrogradation, or recrystallization, of the starch components of flour. The major components of starch are amylose, which forms the core of starch granules, and amylopectin, which provides an outer "envelope". Retrogradation of starch suspensions on standing, observed as the precipitation of the amylose component, has been reported, which, by some, has been given as the explanation for staling. Others have proposed that the formation of associations between the amylopectin chains lead to a rigidity of bread crumb structure which is characteristic of stale bread (cf, Kulp, K., and Ponte, J.G.,Jr. 1981. CRC Critical Reviews in Food Science and Nutrition 15:1-48; Food Science and Technology, Vol. 15, Eliasson, A-C., and Larsson, K. eds. Marcel Dekker, New York, 1993).

Accordingly, use of various starch modifiers, such as emulsifiers and hydrating agents, has attracted much interest. Retardation of the staling process may be achieved, for example, by the addition of monoglycerides to dough. The antistaling effect of the monoglycerides may be attributed to their ability to bind water as well as to the formation of

monoglyceride-amylose complexes. It is believed that these long hydrocarbon chains can penetrate into the cavity of the amylose helix and thereby stabilise the helical structure to prevent retrogradation. However, emulsifiers and hydrating agents have the disadvantage of significantly altering the taste of the product in question.

Amylolytic enzymes, such as endo- or exo-acting glucanases, e.g., α- or β-amylase or maltogenic α-amylase, and glucoamylase have been shown to prevent starch retrogradation, *cf*, JP 62-79745 (Showa Sangyo Co. Ltd.) , EP 412 607 (Gist-Brocades NV) and EP 494 233 (Novo Nordisk A/S), and do not alter product flavour.

It is generally known that efficient enzymatic degradation requires a tight interaction between the enzyme and its substrate. In the case of glucoamylases, for example, physicochemical studies have revealed a modular organization consisting of a domain defining the active site for substrate hydrolysis covalently linked to a discrete carbohydrate binding domain (Coutinho, P.M., and Reilly, P.J. 1994. Protein Engineering 7:393-400). The presence of functionally independent catalytic and binding domains is typical of many polysaccharidases involved in the hydrolysis of insoluble, highly structured cellulosic and hemicellulosic matrices. These binding domains occur predominantly at the N and C termini of enzymes, but a few are found internally (reviewed by Tomme, P., et al. in Enzymatic Degradation of Insoluble Carbohydrates, John N. Saddler and Michael H. Penner, eds., ACS Symposium Series, No. 618, 1996, chapter 10). Ong et al. (1989, TIB Tech 7, p. 239-243, and WO 93/21331) also describe a number of cellulose binding domains.

The precise roles of these binding domains in hydrolysis and their mechanisms of binding to carbohydrates are not well understood. Several studies have clearly shown that removal of the carbohydrate binding domain results in reduced catalytic activity. It has been suggested that the binding domain may enhance catalytic activity by increasing the local enzyme concentration on its substrate surface (Nidetzky, B., et al., 1994. Biochemical Journal 303:817-23) or it could be involved in the disruption of noncovalent interactions to increase substrate accessibility (Din, N., et al., 1991. Archives of Microbiology 153:129-33; Din, N., et al., 1994. Molecular Microbiology 11:747-755).

BRIEF DISCLOSURE OF THE INVENTION

It has been found surprisingly that the combined use of a carbohydrate binding domain (CBD) and an anti-staling enzyme in the preparation of a baked product, in particular bread, can significantly enhance the anti-staling effect obtained with the anti-staling enzyme when used alone. The present invention is based on this finding.

Accordingly, in a first aspect the present invention relates to a bread improving composition comprising an effective amount of a carbohydrate binding domain and an effective amount of an anti-staling enzyme. The bread improving composition is of particular interest for the production of a baked food item or other starch based food item, such as pasta, with a retarded rate of staling.

The term "carbohydrate binding domain" (CBD) is defined and exemplified in the "Detailed Description of the Invention" section further below.

As used herein, the term "an anti-staling enzyme" refers to an enzyme that exhibits activity which can prevent or retard the staling of baked food items, in particular, bread and related products. In principle, staling is deterioration in the qualities of such food items resulting in a product which is unpalatable to the consumer. There are many changes in the crumb properties characteristic of staling (reviewed by Herz, K.O. 1965. Food Technol. 19:1828). These include loss of crumb moisture and elasticity; a decrease in soluble starch; increased crust moisture, crumbliness, starch crystallinity, opacity and firmness; and loss of flavour. Examples of enzymes which have been used to retard staling have been presented above in the "Background of the Invention".

In the present context the term "bread improving composition" is intended to indicate a composition which, in addition to the combination of a CBD and an anti-staling enzyme, may comprise other substances conventionally used in baking to provide desirable qualities of baked products. Examples of such components are given below. In addition to bread, the term "baked products" is intended to include rolls, baguettes, certain kinds of cakes, muffins, buns, and the like as discussed in further detail in the "Detailed Description of the Invention" section below.

The term "an effective amount" is intended to indicate an amount of a CBD, which in

combination with an "effective amount" of an enzyme with anti-staling activity, is sufficient for providing a discernible effect on at least one of the properties believed to contribute to staling as defined above and in the first paragraph of the section "Detailed Disclosure of the Invention" below.

In a second aspect, the present invention relates to a method of preparing a baked product with a retarded rate of staling, in which the method comprises incorporating into the bread dough or dough ingredients an effective amount of a carbohydrate binding domain as defined herein in combination with an effective amount of an anti-staling enzyme and subjecting the resultant dough to baking under suitable conditions.

In still further aspects, the present invention relates to a dough and a baked product, respectively, as well as to a pre-mix produced by the present method which comprises an effective amount of a carbohydrate binding domain as defined herein in combination with an anti-staling enzyme.

In the present context, the term "pre-mix" is intended to be understood in its conventional meaning, i.e. as a mixture of baking agents, normally including flour, which has been prepared to permit storage under designated conditions and provide convenience in handling during dough preparation processes. Such a pre-mix may be of advantageous use in industrial and commercial bread-baking plants and facilities, as well as in retail bakeries.

In a final aspect, the present invention relates to the use of an effective amount of a carbohydrate binding domain as defined herein in combination with an anti-staling enzyme for preventing or reducing the staling of baked products, in particular, bread.

DETAILED DISCLOSURE OF THE INVENTION

Carbohydrate-binding domains

A carbohydrate-binding domain (CBD) is a polypeptide amino acid sequence which binds preferentially to a polysaccharide (carbohydrate), frequently, although not necessarily exclusively, to a water-insoluble (including crystalline) form thereof. Although a number of types of CBDs have been described in the patent and scientific literature, the majority, which are derived from cellulolytic enzymes, i.e., cellulases, are commonly referred to as "cellulose-binding domains". A typical cellulose-binding domain will thus be a CBD which

occurs in a cellulase. For example, US Patent 5,496,934 and Goldstein, M.A., et al (1993, Journal of Bacteriology 175:5762-8) describe a CBD from *Clostridium celluvorans* which demonstrates a high affinity for crystalline cellulose, and is commercially available (cf., Sigma, USA).

Likewise, other sub-classes of CBDs would embrace, e.g., chitin-binding domains (CBDs which typically occur in chitinases), xylan-binding domains (CBDs which typically occur in xylanases), mannan-binding domains (CBDs which typically occur in mannanases), and the like.

CBDs are found as integral parts of large polypeptides or proteins consisting of two or more polypeptide amino acid sequence regions, i.e., functional domains, especially in hydrolytic enzymes (hydrolases) which typically comprise a catalytic domain containing the active site for substrate hydrolysis and a carbohydrate-binding domain (CBD) for binding to the carbohydrate portion of the substrate in question. Such enzymes can comprise more than one catalytic domain and one, two or three CBDs, and optionally further comprise one or more polypeptide amino acid sequence regions linking the CBD(s) with the catalytic domain(s), a region of the latter type usually being denoted a "linker". Examples of hydrolytic enzymes comprising a CBD, some of which have already been mentioned above, are cellulases, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. CBDs have also been found in algae, e.g., the red alga *Porphyra purpurea,* in the form of a non-hydrolytic polysaccharide-binding protein (see P. Tomme, et al., "Cellulose-Binding Domains - Classification and Properties" in Enzymatic Degradation of Insoluble Carbohydrates, John N. Saddler and Michael H. Penner, Eds., ACS Symposium Series, No. 618, 1996). However, most of the known CBDs, which are classified and referred to by P. Tomme et al. (*op cit.*) as "cellulose-binding domains", are derived from cellulases and xylanases.

In the present context, the term "cellulose-binding domain" is intended to be understood in the same manner as in the latter reference (P. Tomme, et al., *op. cit*), and the abbreviation "CBD" as employed herein will thus often be interpretable either in the broader sense as a carbohydrate-binding domain or in the (in principle) narrower sense as a cellulose-binding domain. The P. Tomme, et al., reference classifies more than 120 "cellulose-binding domains" into 10 families (I-X) which may have different functions or roles in connection with the mechanism of substrate binding. However, it is anticipated that

new family representatives and additional CBD families will be identified in the future.

In proteins and polypeptides in which CBDs occur, e.g. enzymes, typically hydrolytic enzymes, a CBD may be located at the N or C terminus, or at an internal position.

That region of a polypeptide or protein, e.g., a hydrolytic enzyme, which constitutes a CBD *per se* typically consists of more than about 30 and less than about 250 amino acid residues. For example, those CBDs listed and classified in Family I in accordance with P. Tomme, et al. (*op. cit.*) consist of 33-37 amino acid residues, those listed and classified in Family IIa consist of 95-108 amino acid residues, those listed and classified in Family VI consist of 85-92 amino acid residues, whereas one CBD, derived from a cellulase from *Clostridium thermocellum*, listed and classified in Family VII consists of 240 amino acid residues. Accordingly, the molecular weight of an amino acid sequence constituting a CBD *per se* will typically be in the range of from about 4kDa to about 40kDa, and usually below about 35kDa.

Cellulases and cellulase genes useful for preparation of CBDs

Techniques suitable for isolating a cellulase gene are well known in the art. In the present context, the term "cellulase" refers to an enzyme which catalyses the degradation of cellulose to glucose, cellobiose, triose and/or other cello-oligosaccharides.

Preferred cellulases, i.e. cellulases comprising preferred CBD's, in the present context are microbial cellulases, particularly bacterial or fungal cellulases. Endoglucanases (EC 3.2.1.4), particularly monocomponent (i.e., recombinant) endoglucanases, are a preferred class of cellulases.

Useful examples of bacterial cellulases are cellulases derived from or producible by bacterial strains from the group consisting of *Pseudomonas, Bacillus, Cellulomonas, Clostridium, Microspora, Thermotoga, Caldocellum* and *Actinomyces,* such as *Streptomyces, Termomonospora* and *Acidothemus*, in particular from the group consisting of *Pseudomonas cellulolyticus, Bacillus lautus, Cellulomonas fimi, Clostridium thermocellum, Microspora bispora, Termomonospora fusca, Termomonospora cellulolyticum* and *Acidothemus cellulolyticus*.

The cellulase may be an acid, a neutral or an alkaline cellulase, i.e. exhibiting maximum cellulolytic activity in the acid, neutral or alkaline pH range, respectively.

A useful cellulase is an acid cellulase, preferably of fungal origin, which is derived from or

producible by fungal strains from the group of genera consisting of *Trichoderma, Myrothecium, Aspergillus, Phanaerochaete, Neurospora, Neocallimastix* and *Botrytis*.

A preferred useful acid cellulase is one derived from or producible by fungal strains from the group of species consisting of *Trichoderma viride, Trichoderma reesei, Trichoderma longibrachiatum, Myrothecium verrucaria, Aspergillus niger, Aspergillus oryzae, Phanaerochaete chrysosporium, Neurospora crassa, Neocallimastix partriciarum* and *Botrytis cinerea.*

Another useful cellulase is a neutral or alkaline cellulase, preferably a fungal neutral or alkaline cellulase, which is derived from or producible by fungal strains from the group of genera consisting of *Aspergillus, Penicillium, Myceliophthora, Humicola, Irpex, Fusarium, Stachybotrys, Scopulariopsis, Chaetomium, Mycogone, Verticillium, Myrothecium, Papulospora, Gliocladium, Cephalosporium* and *Acremonium.*

A preferred alkaline cellulase is one derived from or producible by a fungal strain from the group of species consisting of *Humicola insolens, Fusarium oxysporum, Myceliopthora thermophila, Penicillium janthinellum* and *Cephalosporium* sp., preferably from the group of species consisting of *Humicola insolens* DSM 1800, *Fusarium oxysporum* DSM 2672, *Myceliopthora thermophila* CBS 117.65, and *Cephalosporium* sp. RYM-202.

A preferred cellulase is an alkaline endoglucanase which is immunologically reactive with an antibody raised against a highly purified 43kDa endoglucanase derived from *Humicola insolens* DSM 1800, or which is a derivative of the latter 43kD endoglucanase and exhibits cellulase activity.

Other examples of useful cellulases are variants of parent cellulases of fungal or bacterial origin, e.g. a parent cellulase derivable from a strain of a species within one of the fungal genera *Humicola*, *Trichoderma* and *Fusarium.*

Other proteins and protein genes useful for preparation of CBDs

Examples of other types of hydrolytic enzymes which comprise a CBD are, as already mentioned, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. Also as mentioned previously, CBDs have been found, for example, in certain algae, such as in the red alga *Porphyra purpurea* in the form of a non-hydrolytic polysaccharide-binding protein. Reference may be made to P. Tomme, et al. (*op cit.*) for further details concerning sources, such as organism genera and species, of such CBDs.

CBDs derived from such sources will also be generally be suitable for use in the context of

the invention. In this connection, techniques suitable for isolating, e.g., xylanase genes, mannanase genes, arabinofuranosidase genes, acetylesterase genes or chitinase genes are well known in the art.

Isolation of a CBD

In order to isolate a cellulose-binding domain of, e.g., a cellulase, any of several genetic engineering approaches may be used. For example, one method uses restriction enzymes to remove a portion of the gene, then fusing the remaining gene-vector fragment in frame to obtain a mutated gene that encodes a protein truncated for a particular gene fragment. Another method involves the use of exonucleases such as *Ba*131 to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene. These gene-deletion methods result in a mutated gene encoding a shortened molecule in which the expressed product may then be evaluated for substrate binding, e.g. cellulose-binding, ability. Appropriate substrates for evaluating the binding ability include cellulosic materials such as Avicel® (microcrystalline cellulose; Fluka Chemie AG, CH) and cotton fibres.

Single Carbohydrate Binding Domain (single CBD)

The CBD to be used in the present invention may be a "single carbohydrate binding domain (CBD)" or "isolated CBD" or "separated CBD"; said terms may be used interchangeably. The single CBD may include up to the entire region of the amino acid sequence of a CBD-containing protein, e.g. an enzyme such as a polysaccharide hydrolysing enzyme, which is essentially free of the catalytic domain, whilst retaining the CBD function. Thus, in the context of the invention, the entire amino acid sequence comprising the catalytic function of a cellulolytic enzyme, i.e., a cellulase, or other enzymes comprising one or more CBDs is not to be regarded as a single CBD.

Typically a single CBD constitutes a CBD of a polysaccharide hydrolysing enzyme, the CBD of a cellulose binding protein or a protein designed and/or engineered to be capable of binding to a cellulosic carbohydrate.

The single CBD is at least as large as the minimum number of amino acids in a sequence required to bind to cellulosic carbohydrates. A single CBD may also be an amino acid sequence in which the binding and catalytic domain are one and the same.

Anti-staling enzymes

The anti-staling enzyme to be used in accordance with the present invention is preferably an amylolytic enzyme, i.e. an enzyme capable of degrading or othewise modifying starch or starch components. Examples of amylolytic enzymes to be used in the present invention include amylases without any α-1,4-endo-activity, such as an α-1,4-exoglucanase or an α-1,6-endoglucanase, e.g., α-amylase, a β-amylase, an amyloglucosidase, a maltogenic α-amylase, a cyclodextrin glucanotransferase (CGTase), and the like.

It is presently contemplated that the origin of the anti-staling enzyme to be used in the present invention is not critical as long as the enzyme in question has the properties mentioned above. Thus, the anti-staling enzyme may be of any origin, including mammalian, plant, and microbial (including fungal and bacterial) origin.

The anti-staling enzyme to be used in the present invention may be obtained from its naturally occurring source, such as a plant or organism, or relevant part thereof, by any suitable technique, and in particular by use of recombinant DNA techniques as known in the art (cf., Sambrook, J., et al, Molecular Cloning, Vol. 1-3, A Laboratory Manual. 1989, Cold Spring Harbor Press, Cold Spring Harbor NY, USA). The use of recombinant DNA techniques normally comprises transforming a host cell with a recombinant DNA vector consisting of the gene of interest inserted between an appropriate promoter and terminator, then cultivating the host cell in a culture medium under conditions permitting the expression of the enzyme and recovering the enzyme from the culture. The DNA sequence may be of genomic, cDNA or synthetic origin or any mixture of these, and may be isolated or synthesised in accordance with methods known in the art.

Of particular interest is the maltogenic α-amylase commercially available from Novo Nordisk A/S as Novamyl®; the antistaling agents Stalingase™ available from Gist-brocades N.V.; Grindamyl MaxLife™ and other products of the product line Grindamyl™ available from Grindsted Products; enzyme products of the product line Veron™ available from Röhm GmbH; plant β-amylases, e.g. from soy bean, wheat, barley, commercially available as Nagase and Speezyme (Genencor Inc.).

In addition the anti-staling enzyme may be a glucose oxidase, e.g. the glucose oxidase available from Novo Nordisk A/S as Gluzyme®; or a lipase, e.g. the lipase available from Novo Nordisk A/S as Novozym® 677.

It is presently believed that the use of the carbohydrate binding domain in accordance with the present invention provides a reduced staling effect as measured by, e.g. crumb firming, loss of crumb elasticity, reduced slice-ability, reduced palatability or reduced flavour.

It is further contemplated that favourable results may also be obtained when the CBD and the anti-staling enzyme are further combined with other enzymes. Therefore, the bread improving composition of the invention may comprise one or more additional enzymes, wherein such additional enzyme(s) may be added together with the CBD and the anti-staling enzyme according to any of the methods of the invention.

Examples of other enzymes include cellulases and hemicellulases, e.g., a pentosanase, such as xylanase, which can partially hydrolyse pentosans to produce an increase in dough extensibility, improve dough stability and/or increase loaf volume; glycosyltransferases, in particular, a 1,4-α-glucan branching enzyme (E.C. 2.4.1.18); lipases, e.g., a phospholipase, to soften the dough by modifying lipids present in the dough or dough constituents; oxidoreductases, e.g., a glucose oxidase, a pyranose oxidase, a lipoxygenase, a peroxidase, a laccase, or an L-amino acid oxidase, to improve dough consistency; proteases to provide gluten weakening, in particular when hard wheat flour is used; peptidases, in particular, an exopeptidase for flavour enhancement; and transglutaminases. These other enzyme components may be of any origin, and preferably of microbial origin. They may be obtained by conventional techniques used in the art such as by recombinant DNA techniques or by isolation from a cultured microorganism which in nature produces the enzyme in question.

The enzyme(s) and the CBD to be used in the present invention may be in any form suitable for the use in question, e.g. in the form of a dry powder or granulate, in particular a non-dusting granulate, a liquid, in particular a stabilised liquid, or a protected enzyme. Granulates may be produced, e.g. as disclosed in US 4,106,991 and US 4,661,452 (both to Novo Industri A/S), and may be optionally coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilised by adding nutritionally acceptable stabilisers such as a sugar, a sugar alcohol or another polyol, lactic acid or another organic acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238 216.

Normally, for inclusion in pre-mixes or flour it is advantageous that the enzyme(s) and

the CBD are in the form of a dry product, e.g. a non-dusting granulate, whereas for inclusion together with a liquid it is advantageously in a liquid form.

In addition, or as an alternative, to other enzyme components, the bread improving composition may comprise another dough and/or bread improving agent conventionally used in baking, e.g. one or more of the following constituents: proteins (such as milk powder to provide crust colour); gluten (to improve the gas retention power of weak flours); soy (to provide additional nutrients and improve water binding); an emulsifier (to improve dough extensibility and, to some extent, the consistency of the resulting bread); granulated fat (to soften the dough and improve the texture of the bread); an oxidant, e.g. ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA), calcium peroxide, and ammonium persulfate (to strengthen the gluten structure); an amino acid, e.g. L-cysteine (to improve mixing properties); a sugar; a salt, e.g. sodium chloride, calcium acetate, sodium sulfate and calcium sulphate (to make the dough firmer); flour; and starch. Such components may also be added directly to the dough in accordance with a method of the invention.

Examples of suitable emulsifiers are mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, phospholipids, lecithin and lysolecithin.

The bread improving composition of the invention is typically included in the dough in an amount corresponding to 0.01-5%, more specifically between 0.1-3%.

In accordance with the method of the invention, the enzyme and the CBD may be added, either separately or concurrently, to the mixture from which the dough is made or to any ingredient, e.g. flour, from which the dough is to be made. Alternatively, the enzyme and the CBD may be added, either separately or concurrently, as a constituent of a bread improving composition as described above, either to flour or other dough ingredients, or directly to the mixture from which the dough is to be made.

The dosage of the enzyme and the CBD, respectively, to be used in the method of the present invention should be adapted to the nature of both the dough and the baked product in question, including the composition of the dough and the processes used in its preparation and baking, as well as to characteristics specific to the enzyme to be used. Normally, the enzyme preparation is added in an amount corresponding to 0.01-1000 mg enzyme protein per kg of flour, preferably 0.1-100 mg enzyme protein per kg of flour, ideally 0.1-10 mg

enzyme protein per kg of flour. The CBD may be preferably added at a concentration in the range from 0.001-1000 mg per kg of flour, more preferably 0.01-100 mg per kg of flour, and most preferably 0.05-50 mg per kg of flour.

The desirable level of enzyme activity and CBD, respectively, to be used in the connection with the present invention will depend on characteristics specific to the enzyme and CBD and the enzyme/CBD substrate in question. The skilled person may determine a suitable dosage of enzyme activity and CBD activity on the basis of methods known in the art. Preferably, the dosage is evaluated by means of the anti-staling effect provided and other effects (such as volume, dough characteristics, and the like) which should be at an acceptable level.

When one or more additional enzyme activities are to be added in accordance with the method of the invention, these activities may be added separately or concurrently with the CBD, optionally as constituent(s) of the bread improving composition of the invention. The other enzyme activities may be any of the above described enzymes and may be dosed in accordance with established baking practice.

As mentioned above, the CBD and the anti-staling enzyme, optionally in combination with one or more second enzymes as described above, is added to any mixture of dough ingredients, to the dough, or to any of the ingredients to be included in the dough. In other words, the CBD and the anti-staling enzyme may be added in any step of the dough preparation and may be added in one, two or more steps where appropriate.

The handling of the dough and/or baking is performed in any suitable manner for the dough and/or baked product in question, typically including the steps of kneading the dough, subjecting the dough to one or more proofing treatments, and baking the dough under suitable conditions, i.e. at a suitable temperature and for a sufficient period of time. For instance, the dough may be prepared by using a normal straight dough process, a sour dough process, an overnight dough method, a low-temperature and long-time fermentation method, a frozen dough method, the Chorleywood Bread process, or the Sponge and Dough process.

The dough and/or baked product prepared by the method of the invention are normally based on wheat meal or flour, optionally in combination with other types of meal or flour such as corn flour, rye meal, rye flour, oat flour or meal, soy flour, sorghum meal or flour, or potato meal or flour.

In the present context the term "baked product" is intended to include food prepared

from a dough or a batter, either of a soft or a crisp character, and which is cooked by baking. Examples of baked products, whether of a white, light or dark type, which may be advantageously produced by the present invention are bread (in particular white, whole-meal, rye and mixtures thereof), typically in the form of loaves or rolls, French baguette-type bread, bagels, pita bread, tacos, tortillas, cakes, pannetone, pan-cakes, biscuits, crisp bread, pizza and the like.

The dough of the invention may be of any of the types discussed above, and may be fresh, frozen or par-baked. The preparation of frozen dough is described by K. Kulp and K. Lorenz in "Frozen and Refrigerated Doughs and Batters."

From the above disclosure it will be apparent that the dough of the invention is normally a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways such as by adding sodium bicarbonate or the like or by adding a leaven (fermenting dough), but it is preferred to leaven the dough by adding a suitable yeast culture such as a culture of *Saccharomyces cerevisiae* (baker's yeast). Any of the commercially available *S. cereviciae* strains may be employed.

As mentioned above, the present invention further relates to a pre-mix, e.g., in the form of a flour composition, of dough and/or baked products made from dough, in which the pre-mix comprises a CBD and optionally other enzymes as specified above. The pre-mix may be prepared by mixing enzyme preparation(s) comprising the relevant enzyme(s) and/or a bread improving composition of the invention comprising the enzyme(s) and the CBD with a suitable carrier such as flour, starch, a sugar or a salt. The pre-mix may contain other dough and/or bread improving additives, e.g., any of the additives, including enzymes, mentioned above.

In a final aspect the invention relates to the use of a CBD, preferably in combination with an anti-staling enzyme, for the preparation of pasta dough, preferably prepared from durum flour or a flour of comparable quality. The dough may be prepared by use of conventional techniques and the CBD and anti-staling enzyme used at a dosage similar to that described above. The CBD and the anti-staling enzyme may be as described herein above.

Techniques which can be used to determine improvements achieved by use of the present invention are described below. The organoleptic qualities mentioned above may be evaluated using procedures well established in the baking industry, and may include, for

example, the use of a panel of trained taste-testers.

EXAMPLES

The invention is further illustrated with reference to the following example which should not in any way be construed as limiting the scope of the invention as defined in the appended claims.

MATERIALS AND METHODS

The following were used in Examples 1 and 2.

Transformation Host Strains:

B.subtilis PL2306 was used as a transformation host strain. It is a cellulase-negative strain developed by introducing a disruption in the transcriptional unit of the known *Bacillus subtilis* cellulase gene in *B.subtilis* strain DN1885(Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjøholm, C. (1990) Cloning of *aldB*, which encodes alpha-acetolactate decarboxylase, an exoenzyme from *Bacillus brevis*. J. Bacteriol. 172:4315-4321). Not only was the cellulase gene of DN1885 disrupted but also two protease encoding genes where disrupted, namely *aprE* (Stahl,M.L., and Ferrari, E. 1984. J. Bacteriol. 158:411-418) and *nprE* (Yang, M.Y., et al., 1984. J. Bacteriol. 160:16-21).

The disruption was performed essentially as described in Bacillus subtilis and other Gram-Positive Bacteria; A.L. Sonenshein, J.A. Hoch and Richard Losick, Eds. American Society for Microbiology, 1993, p.618.

A strain, ToC46, developed from *Bacillus subtilis* was used as a secondary expression host. The construction of ToC46 is described by Diderichsen, B., et al. (1990. J. Bacteriol., 172:4315-4321). Transformation of competent cells was performed as described above.

Plasmid:

pMB100 is a derivative of pDN1528 (Jørgensen, S., et al. (1991) Journal of Bacteriology 173:559-567). The plasmid is essentially the same as pDN1528 but differs by the introduction of a SacI site between the stop codon of the *amyL* gene (Jørgensen, S., et

al. (1991) Journal of Bacteriology 173:559-567) and its terminator for cloning purposes.

Solutions and Media:

TY and LB agar were prepared as described in Current Protocols in Molecular Biology, Ausubel, F.M., et al., Eds. John Wiley and Sons, 1995.

SB was prepared from 32 g tryptone, 20 g yeast extract, 5 g NaCl and 5 ml 1 N NaOH, mixed together in sterile water to a final volume of 1 litre. The solution was sterilised by autoclaving for 20 min at 121°C.

10% Avicel® was prepared from 100 g of Avicel® (microcrystalline cellulose; Fluka Chemie AG, CH) in sterile water to a final volume of 1 litre, and sterilised by autoclaving for 20 min at 121°C.

General molecular biology methods:

DNA manipulations and transformations were performed using standard methods of molecular biology as described, for example in Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989; Ausubel, F.M., et al., *op. cit.*; Molecular Biological Methods for *Bacillus*, Harwood, C.R., and Cutting, S. M., Eds. John Wiley and Sons, 1990.

Enzymes for DNA manipulations were used according to the specifications provided by the respective suppliers.

Isolation of genomic DNA:

Clostridium stercorarium NCIMB 11754 was grown anaerobically at 60°C in specified media as recommended by The National Collections of Industrial and Marine Bacteria Ltd. (Scotland). Cells were havested by centrifugation.

Genomic DNA was isolated as described by Pitcher et al. (1989; Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8:151-156).

Example 1: Cloning and Expression of a CBD-dimer from *Clostridium stercorarium* XynA

The CBD-dimer of *Clostridium stercorarium* (NCIMB 11754) of xylanase A (XynA)

(GenBank and SWISS-PROT Accession No.13325) was expressed in a strain of *B. subtilis*.

The SWISS-PROT data describes the position of the putative cellulose binding domains, which was used to design specific PCR primers. Designed into the primers at the same time were extra codons corresponding to amino acids downstream of the signal sequence of *amyL* in order to enable secretion of the CBDs.

In vitro amplification of the CBD-dimer of *Clostridium stercorarium* XynA

Approximately 100 to 200 ng of genomic DNA was PCR- amplified in HiFidelity™ PCR buffer (Boehringer Mannheim, DE) supplemented with 200 μM of each dNTP, 2.6 units of HiFidelity™ Expand enzyme mix, and 300 pmol of each of the following primers:

CLOST03U:

5'- CTG CCT CAT TCT GCA GCA GCG GCG GCA AAT CTT AAT CCA ACT CCT GCC CCA TCT CAA AGC -3'

CLOST03D2:

5'- CTG CCT CAT TGC ATG CAG AGC TCC TAC TAC CAG TCA ACA TTA ACA GGA CCT GAG-3'

Restriction sites PstI in CLOST03U and SacI in CLOST03D2 are underlined.

The PCR reactions were performed using a DNA thermal cycler (Landgraf, DE). The reaction cycle was set for one incubation at 94°C for 2 min, 30 sec at 60°C and 45 sec at 72°C followed by ten cycles of PCR performed using a cycle profile of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 45 sec and twenty cycles of denaturation at 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec plus 20 seconds added per cycle. Ten-μl aliquots of the amplification product were analysed by electrophoresis in 1.5 % agarose gels (NuSieve, FMC) with ReadyLoad 100bp DNA ladder (GibcoBRL, DK) as a size marker.

Cloning by polymerase chain reaction (PCR): Subcloning of PCR fragments

Fourty-μl aliquots of the PCR products generated as described above were purified using QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's

instructions. The purified DNA was eluted in 50 μl of 10mM Tris-HCl, pH 8.5. Twenty five-μl of the purified PCR fragment were digested with SacI and PstI, then electrophoresed in 1.5% low gelling temperature agarose gels (SeaPlaque GTG, FMC). The relevant fragments were excised from the gels and purified using the QIAquick Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to a PstI/SacI digested pMB100 plasmid, and the ligation mixture was used to transform *B.subtilis* PL2306.

Identification and charaterization of positive clones

Cells were plated on LBPG-chloramphenicol agar plates (LB agar plates containing chloramphenicol (6 μg/ml), 0.4% glucose and 10mM potassium hydrogen phosphate), and incubated at 37°C overnight. Colonies were restreaked the next day onto fresh LBPG-chloramphenicol agar plates and incubated at 37°C overnight. The next day single colonies were transferred to liquid LB medium containing chloramphenicol (6 μg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using the Qiagen Plasmid Purification Mini Kit (Qiagen, USA) according to the manufacturer's instructions. However, the recommended resuspension buffer was supplemented with 1 mg/ml of chicken egg white lysozyme (Sigma, USA) prior to lysing the cells at 37°C for 15 min. Five-μl samples of the plasmids were digested with PstI and SacI, then analysed by gel electrophoresis on a 1.5% agarose gel (NuSieve, FMC) for DNA fragments of the same size as seen in the PCR amplification. The clone MB203 was selected.

Nucleotide sequencing the cloned DNA fragment

Qiagen purified plasmid DNA was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) using the same primers used in the PCR amplification in an Applied Biosystems 373A automated sequencer, according to the manufacturer's instructions. Analysis of the sequence data was performed according to Devereux, J., et al. (1984. Nucleic Acids Research 12:387-395).

Expression, secretion and functional analysis of the cloned CBDs

MB203 was incubated for 20 hours in SB broth at 37°C with shaking at 250 rpm. A

one-ml aliquot of cell-free supernatant was mixed with 200 µl of 10% Avicel®. The mixture was incubated for 1 hour at 0°C to allow binding of the CBD in solution to the Avicel®. The Avicel® was centrifuged for 5 min at 5000 g; the pellet was resuspended in 100 µl of SDS-page buffer, boiled at 95°C for 5 min and centrifuged again at 5000g for 5 min. Twenty-five µl of the boiled solution was loaded onto a 18% Laemmli Tris-glycine, SDS-PAGE NOVEX gel (Novex, USA), then electrophoresed in a Xcell™ Mini-Cell (NOVEX, USA) as recommended by the manufacturer. All subsequent handling of gels, including comassie staining and destaining and drying were performed as described by the manufacturer.

The appearance of a protein band at approximately 35 kDa indicated expression in *B.subtilis* of functional CBDs.

Example 2: Expression and purification of the XynA CBD-dimer cloned from *C.stercorarium*

The plasmid in MB203 was isolated and used to transform another *Bacillus subtilis* strain, ToC46, resulting in a new CBD-dimer expressing clone called MB206. Using this strain as the expression host for the CBD-dimer, the clone was incubated in shake flasks containing SB media with chloramphenicol at 6 µg/ml for 20 hours, at 37°C and shaking at 250 rpm.

1400 ml of culture fluid supernatant was cooled in an ice bath, then filtered through Whatman Glass filter F and further sterile filtered through 0.45 micron Millipore Type HVLP filter.

Fifty grams of Avicel® were equilibrated in 0.1 M sodium phosphate buffer, pH 7.5, at room temperature for 30 min., following which the supernatant was removed and the Avicel® slurry was cooled to 4°C. The filtered culture supernatant was mixed with the Avicel® slurry at 4°C for 30 min., then allowed to settle for 10 min before the supernatant was removed. The Avicel®-protein complex was packed in a column and washed with 0.1M sodium phophate buffer, followed by a wash with the same buffer containing 0.5M sodium chloride. Finally, the CBD was eluted using deionized water.

The CBD was eluted in a total volume of 78 ml. Sodium chloride crystals were added for a final concentration of 0.5M before the CBD solution was concentrated in an Amicon

cell with a R81P membrane which has a molecular weight cut-off of 8 kDa.

The CBD solution was concentrated to 30 ml and gave an absorbance at 280 nm of 1.2. The molar extinction coefficient of MB206 is 42000. Thus, the protein concentration was calculated to be 0.82 mg/ml, and a total of 25 mg of highly purified CBD dimer was recovered. The final purified product revealed only a single band by SDS-PAGE analysis.

Example 3: Measurement of Amylopectin Retrogradation

The effect of a CBD on amylopectin retrogradation was assayed by differential scanning calorimetry (DSC). 730 mg of a solution of 50% amylopectin (Sigma, USA) in 0.1M sodium acetate, pH 5.5, was added to six Hart Scientific DSC sample ampules (Hart Scientific, Pleasant Grove UT, USA). One of the following was added to each ampule: 20 ml Novamyl® (10 MANU/ml); 20 ml CBD alone (Sigma, 0.1 mg/ml; or MB206, 0.82 mg/ml); 20 ml [Novamyl® (10 MANU/ml) + CBD (Sigma 0.1 mg/ml or MB206, 0.82 mg/ml)]; or 20 ml H_2O. The samples were sealed and incubated at 37 °C for 1 hour, then gelatinised by treating at 100°C for 1 hour, before storage for 7 days at room temperature. A DSC scan was then performed in the Hart Scientific Differential Scanning Calorimeter using a constant scan rate of 90° per hour from 5 to 95°C. Data were analysed using the MicroCal Origin software (MicroCal, Inc., Northampton MA, USA). The melting enthalpies (area under the observed melting peak) were used for quantifying the relative amount of retrogradation of amylopectin.

The enzyme unit MANU is as defined in EP 494 233.

As can be observed from Table 1 below, Novamyl® in the absence of CBD results in a decrease in the amount of retrogradation, whereas the CBD alone has no effect. However, when CBD is added to Novamyl®, the relative decrease in retrogradation is enhanced.

Sample	Retrogradation (relative units)
no enzyme	1
2.7 μg/ml CBD (Sigma)	1.01
2.0 μg/ml CBD (MB206)	0.98
0.27 MANU/ml Novamyl®	0.36
2.7 μg/ml CBD (Sigma) + 0.27 MANU/ml Novamyl®	0.19
2.0 μg/ml CBD (MB206) + 0.27 MANU/ml Novamyl®	0.26

Table 1. Retrogradation of amylopectin by various enzyme/CBD treatments.

Example 4: Improved Bread Softness by α-Amylase in Combination with CBD

White bread was prepared using the following basic recipe:

Basic recipe

Wheat flour	100 %
Salt	2 %
Yeast (fresh)	4 %
sugar	3 %
Water	60 %
Shortening	1.5 %
Ascorbic Acid	40 ppm

Novamyl® (300 MANU/kg flour) and CBD (MB206, 7 μg/8 ml), each alone or the combination were added to flour. Neither Novamyl® nor CBD were added to the control flour.

Procedure

1. Dough mixing (Spiral mixer)

   3 min. at 625 RPM

   3.5 min. at 1250 RPM

2. 1st proof: 15 min. at room temperature (about 22 °C), covered by a cloth
3. Scaling and shaping;
4. Final proof: 32°C - 82% RH, 55 min.;
5. Baking: 230°C, 22 min. for rolls and 35 min for loaf.

Evaluation of Baked Products:

Evaluation of staleness and texture may be performed according to AACC method 74-09. Evaluation of softness and elasticity of bread crumb as indicators of bread staling were conducted 0, 3, 6 and 9 days after baking according to the following procedure:

1. A slice of bread was compressed at constant speed in a texture analyser, and the force for compression was measured in g.
2. The firmness of the crumb is measured as the force at 25% compression.
3. The force at 40% compression (P2) and after keeping 40% compression constant for 30 sec. (P3) was measured and the ratio (P3/P2) is defined as the elasticity of the crumb.

The crumb firmness measurements are summarised below in Table 2, where a lower g value indicates a softer; i.e., less stale, bread. CBD appears to have an enhancing effect on Novamyl®. The Day 9 calculated crumb firmness of bread containing the combination is 42% softer than the control, versus 23% for Novamyl® alone.

Day	0	3	6	9
Control	246	1052	1541	2503
Novamyl®	292	801	1142	1925
Novamyl® + CBD	280	774	1161	1458

Table 2. Bread Crumb Firmness (g) by Novamyl®/CBD Treatments.

The results of crumb elasticity evaluation, summarised below in Table 3, indicate that the addition of CBD to Novamyl® has an enhancing effect.

Day	0	3	6	9
Control	70%	55%	50%	49%
Novamyl®	68%	58%	52%	50%
Novamyl® + CBD	68%	58%	53%	52%

Table 3. Bread Crumb Elasticity by Novamyl®/CBD Treatments.

CLAIMS

1. A bread improving composition comprising a carbohydrate binding domain (CBD) and an anti-staling enzyme.

2. A bread improving composition according to claim 1, wherein the CBD is a cellulose binding domain.

3. A bread improving composition according to claim 1 or 2, wherein the CBD is of microbial origin.

4. A bread improving composition according any of claims 1-3, wherein the anti-staling enzyme is an amylolytic enzyme.

5. A bread improving composition according to claim 4, wherein the amylolytic enzyme is an α-amylase, a β-amylase, a maltogenic α-amylase, an amyloglucosidase, or a cyclodextrin glucanotransferase (CGTase).

6. A bread improving composition according to any of claims 1-5, which further comprises an effective amount of one or more of another enzyme such as a cellulase, a hemicellulase, a pentosanase, a lipase, an oxidoreductase, an oxidase, a peroxidase, a protease, a peptidase, or a transglutaminase.

7. A bread improving composition according to any of claims 1-6 in which the enzyme is of microbial origin.

8. A bread improving composition according to any of claims 1-7 which further comprises another bread or dough improving agent.

9. A method of preparing a baked product, which method comprises 1) adding to a dough or dough components an effective amount of a CBD and an anti-staling enzyme; and 2) preparing a baked product from the dough.

10. The method according to claim 9, wherein the CBD and/or anti-staling enzyme is as defined in any of claims 1-8.

11. The method according to claim 9 or 10, wherein the CBD and/or anti-staling enzyme is added in the form of the bread improving composition according to any of claims 1 - 8.

12. The method according to any of claims 9-11, wherein the CBD is added in an amount corresponding to 0.001-1000 mg protein per kg of flour, more preferably 0.01-100 mg protein per kg of flour, most preferably 0.05-50 mg protein per kg of flour.

13. The method according to any of claims 9-12, wherein the anti-staling enzyme is added in an amount corresponding to 0.001-1000 mg protein per kg of flour, more preferably 0.01-100 mg protein per kg of flour, most preferably 0.05-50 mg protein per kg of flour.

14. A baked product such as bread or a dough used in the preparation of a baked product prepared by the method according to any of claims 9-13.

15. A pre-mix for dough used in the preparation of a baked product comprising an effective amount of a CBD and an anti-staling enzyme.

16. Use of an effective amount of a CBD and an effective amount of an anti-staling enzyme for the preparation of a baked product.

17. The use according to claim 16, wherein the CBD is a cellulose binding domain and/or the anti-staling enzyme is an amylolytic enzyme.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00427

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A21D 8/04, A21D 2/26
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, US PATENTS FULLTEXT,CA,MEDLINE,BIOSIS,EMBASE,DBA,FSTA,SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0529712 A1 (QUEST INTERNATIONAL B.V.), 3 March 1993 (03.03.93) --	1-17
X	WO 9523515 A1 (NOVO NORDISK A/S), 8 Sept 1995 (08.09.95), the claims --	1-17
X	EP 0396162 A1 (UNILEVER NV), 7 November 1990 (07.11.90), the claims and page 2, lines 1-4 --	1-17
A	WO 9117244 A1 (NOVO NORDISK A/S), 14 November 1991 (14.11.91), page 1, line 20 - line 21; page 3, line 33 - page 4, line 4 --	1-17

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3512992 A (ALFRED COOKE ET AL), 19 May 1970 (19.05.70), the claims; Example 4 --	1-17
A	Die Stärke, Volume, No 12, 1969, Stig R. Erlander et al, "Explanation of Ionic Sequences in Various Phenomena", page 305 - page 315, page 306, left column, lines 7-12 --	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

07/01/98

International application No.
PCT/DK 97/00427

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